

09/914811 PCT

JC05 Rec'd PCT/PTO 30 AUG 2001

FORM PTO-1390 (REV. 3-90)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEYS DOCKET NO.	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)				16-007	
INTERNATIONAL APPLICATION NO. PCT/GB00/00761		INTERNATIONAL FILING DATE 03 March 2000 (03.03.00)		PRIORITY DATE CLAIMED 05 March 1999 (05.03.99)	
TITLE OF INVENTION GENETIC SCREENING					
APPLICANT(S) FOR DO/EO/US ARMOUR, John Anthony					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items under 35 U.S.C. 371: 1. ■ This express request to immediately begin national examination procedures (35 U.S.C. 371(f)). 2. ■ The U.S. National Fee (35 U.S.C. 371(c)(1)) and other fees as follows:					
CLAIMS	(1) FOR	(2) NUMBER FILED	(3) NUMBER EXTRA	(4) RATE	(5) CALCULATIONS
TOTAL CLAIMS		26 - 20 =	6	x \$18.00	= \$108.00
INDEPENDENT CLAIMS		1 - 3 =		x \$80.00	= \$
MULTIPLE DEPENDENT CLAIMS(S) (if applicable)				+ \$270.00	\$
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(4)): <input type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482)\$690.00 <input type="checkbox"/> No International preliminary examination fee paid to USPTO (37 CFR 1.482) but International search fee paid to USPTO (37 CFR 1.445 (a)(2))..... \$710.00 <input type="checkbox"/> Neither International preliminary examination fee (37 CFR 1.482) nor International search fee (37 CFR 1.445(a)(2)) paid to USPTO..... \$1000.00 <input type="checkbox"/> International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provision of PCT Article 33(2) to(4)..... \$100.00 <input checked="" type="checkbox"/> International search fee prepared by EPO (37 CFR 1.492(a)(5)).....\$860.00					\$860.00
Surcharge of \$130.00 for furnishing the National fee or oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 mos. from the earliest claimed priority date (37 CFR 1.492(e)).					
TOTAL OF ABOVE CALCULATIONS					= \$968.00
■ Reduction by 1/2 for filing by small entity, if applicable. Applicant is entitled to small entity status under 37 CFR 1.27.					\$484.00
SUBTOTAL					= \$484.00
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 mos. from the earliest claimed priority date (37 CFR 1.492(f)).					
TOTAL NATIONAL FEE					\$484.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)) \$40.00					\$
TOTAL FEES ENCLOSED					\$484.00
a. ■ A check in the amount of \$ 484.00 to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed. c. ■ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 23-0630.					

Express Mail Label No.

EL 391522427US

I hereby certify that this paper is being deposited with the
U. S. Postal Service as Express Mail addressed to the
Assistant Commissioner for Patents, Washington, D.C. 20530

8/30/01

Jellen M. Dzylak

3. A copy of the International Application as filed (35 U.S.C. 371(c)(2))
- ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
 - ☒ has been transmitted by the International Bureau.
4. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
5. Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
- ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - ☐ have been transmitted by the International Bureau.
6. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
7. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)) is enclosed.
8. ☐ A translation of the Annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Other document(s) or information included:

9. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
10. ☐ An assignment document for recording.
Please mail the recorded assignment document to:
- ☐ the person whose signature, name & address appears at the bottom of this page.
 - ☐ the following:
11. The above checked items are being transmitted:
- ☐ before the 18th month publication.
 - ☐ after publication and the Article 20 communication but before 20 months from the priority date.
 - ☐ after 20 months but before 22 months (surcharge and/or processing fee included)
 - ☐ after 22 months (surcharge and/or processing fee included)
- Note: Petition to revive (37 CFR 1.137(a) or (b)) is necessary if 35 U.S.C. 371 requirements submitted after 22 months and no proper demand for International Preliminary Examination was made by 19 months from the earliest claimed priority date.
- ☒ by 30 months and a proper demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
 - ☐ after 30 months but before 32 months and a proper demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date (surcharge and/or processing fee included.)
 - ☐ after 32 months (surcharge and/or processing fee included).
- Note: Petition to revive (37 CFR 1.137(a) or (b)) is necessary if 35 U.S.C. 371 requirements submitted after 32 months and a proper demand for International Preliminary Examination was made by 19 months from the earliest claimed priority date.
12. At the time of transmittal, the time limit for amending claims under Article 19
- ☒ has expired
 - ☐ has not yet expired.
13. ☐ Certain requirements under 35 U.S.C. 371 were previously submitted by the applicant on _____, namely:
14. ☐ Enclosed is a verified statement claiming small entity status.
15. ☒ Enclosed is an Amendment Before Office Action.

RICHARD A. SHARPE
NAME

WATTS, HOFFMANN, FISHER & HEINKE CO., L.P.A.
P.O. Box 99839
Cleveland, Ohio 44199-0839


SIGNATURE

34,722
REGISTRATION NO.

09/914811

JC03 Rec'd PCT/TO 30 AUG 2001

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

ARMOUR, John Anthony

Serial No.: N/A

Art Unit: not yet assigned

Filed: Herewith

Examiner: N/A

For: GENETIC SCREENING

Docket: 16-007

Watts, Hoffmann, Fisher & Heinke Co., L.P.A.
PO Box 99839
Cleveland, Ohio 44199-0839

Box Patent Application
Assistant Commissioner of Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

IN THE CLAIMS

Please cancel claim 1 through 26 without prejudice or disclaimer.

Please add the following claims:

27. A method of screening for copy number of target nucleic acid sequences in a sample of genetic material, the method comprising introducing to the sample a plurality of different genetic probes suitable to hybridise with respective target sequences and all flanked by the same or substantially the same primer bonding sites, subjecting the sample to conditions favoring hybridization of the probes to their respective sequences, and amplification of sample-bound probes using a pair of primers, wherein analysis of the respective amounts of amplified probe provides for quantitative determination of the copy number of the respective nucleic acid sequences in the sample.

Express Mail Number: EL391522427 US
I hereby certify that this paper is being deposited with the
U. S. Postal Service as Express Mail addressed to the
Assistant Commissioner for Patents, Washington, D.C. 20231

8/30/01

John M. Byrd

28. A method according to claim 27, characterized in that each probe is distinguished from the other(s).

29. A method according to claim 27, characterized in that each probe is distinguished from the other(s) by having distinguishing mobility characteristics through a separating gel.

30. A method according to claim 27, characterized in that the plurality of different probes comprises a predetermined set of different probes each chosen to be specific for a respective target nucleic acid sequence.

31. A method according to claim 30, characterized in that the set comprises probes suitable to screen a plurality of different nucleic acid sequences simultaneously or substantially simultaneously such that determination of the quantity of each probe product produced enables quantitative determination of the copy number of the respective sequences in the sample.

32. A method according to claim 27, characterized in that the polymerase chain reaction is used to determine the quantity of each probe product produced.

33. A method according to claim 27, characterized in that the method is used to screen sequences of different genes or different sequences within a gene.

34. A method according to claim 27, characterized in that the method is used to screen sequences of different exons in a eukaryotic gene.

35. A method according to claim 27, characterized in that the method is used to detect genetic alterations.

36. A method according to claim 27, characterized in that the method is used to detect genetic deletions (reductions in sequence copy number) or genetic amplification (increases in sequence copy number).

37. A method according to claim 27, characterized in that the genetic material is immobilized prior to hybridization, such that hybridization flanking primers are likewise immobilized.

38. A method according to claim 27, characterized in that an excess of probes is used.

39. A method according to claim 27, characterized in that probes labelled for ready identification are used.

40. A method according to claim 27, characterized in that probes labelled with fluorescent labels are used.

41. A method according to claim 30, characterized in that more than one set of probes is used, either simultaneously or sequentially.

42. A method according to claim 30, characterized in that more than one set of probes is used and flanking primer pairs are the same for each set of probes.

43. A method according to claim 30, characterized in that more than one set of probes is used and flanking primer pairs are different for each set of probes.

44. A method according to claim 27, characterized in that the method comprises means to obviate or mitigate hybridization between primer binding sequences.

45. A method according to claim 27, characterized in that competing oligonucleotides are introduced to the sample.

46. A method according to claim 27, characterized in that competing oligonucleotides are introduced to the sample during the hybridization stage to releasably bind to primer binding sites flanking each probe to mitigate primer binding site interactions.

47. A method according to claim 27, characterized in that unbound probes and primers are thoroughly washed away from bound probes following hybridization and prior to analysis.

48. A method according to claim 27, characterized in that the method is used to screen one or more of DNA, RNA and cDNA with appropriate probe sets.

49. A method according to claim 27, characterized in that the method is used to screen one or more of somatic and germline sequences.


50. A method according to claim 27, characterized in that the method is used to screen for polymorphic alterations.

51. A set of probes adapted for use in performing the method according to claim 27.

52. A kit for performing the method according to claim 27 comprising a probe set, amplification primers and means to enable amplification and analysis of amplification product(s).

Case	Age	Sex	Occupation	Duration of Illness	Site of Lesion	Microscopic Findings	Diagnosis
1	45	M	Farmer	10 years	Right lung	Granulomatous inflammation with caseation	Tuberculosis
2	32	F	Teacher	5 years	Left lung	Granulomatous inflammation with caseation	Tuberculosis
3	58	M	Engineer	15 years	Right lung	Granulomatous inflammation with caseation	Tuberculosis
4	28	F	Student	3 years	Left lung	Granulomatous inflammation with caseation	Tuberculosis
5	65	M	Retired	20 years	Right lung	Granulomatous inflammation with caseation	Tuberculosis
6	40	F	Homemaker	8 years	Left lung	Granulomatous inflammation with caseation	Tuberculosis
7	35	M	Engineer	12 years	Right lung	Granulomatous inflammation with caseation	Tuberculosis
8	50	F	Teacher	18 years	Left lung	Granulomatous inflammation with caseation	Tuberculosis
9	25	M	Student	4 years	Right lung	Granulomatous inflammation with caseation	Tuberculosis
10	60	F	Retired	22 years	Left lung	Granulomatous inflammation with caseation	Tuberculosis

Dated: August 30, 2001


Richard A. Sharpe
Reg. No. 34,722

5

[illegible]

According to the present invention there is provided a method of screening for copy number of target nucleic acid sequences in a sample of genetic material, the method comprising introducing to the sample a plurality of different genetic probes suitable to hybridise with respective target

sequences and all flanked by the same or substantially the same primer binding sites, subjecting the sample to conditions favouring hybridisation of the probes to their respective sequences, and amplification of sample-bound probes using a pair of primers, wherein analysis of the respective amounts of amplified probe provides for quantitative determination of the copy number of the respective nucleic acid sequences in the sample.

Preferably each probe is distinguishable from the other(s), for example by having distinguishing mobility characteristics through a separating gel. Preferably the plurality of different probes comprises a predetermined set of different probes each chosen to be specific for a respective target nucleic acid sequence. The set may comprise probes suitable to screen a plurality of different nucleic acid sequences simultaneously or substantially simultaneously such that determination of the quantity of each probe product produced, preferably using the polymerase chain reaction (PCR), enables quantitative determination of the copy number of the respective sequences in the sample. The method may be used to screen sequences of different genes or different sequences within a gene, such as different exons in a eukaryotic gene. Preferably the method is used to detect genetic alterations such as genetic deletions (reduction in sequence copy number) and genetic amplification (increase in sequence copy number).

Preferably the genetic material is immobilised prior to hybridisation, such that hybridised flanking primers are likewise immobilised. Preferably an excess of probes is used.

Probes labelled for ready identification, such as with fluorescent labels are preferably used. More than one set of probes may be used, either simultaneously or sequentially. The flanking primer pairs may be the same or different for each set of probes.

Preferably the method comprises means to obviate or mitigate hybridisation between primer binding sequences. Competing oligonucleotides

may be introduced to the sample preferably during the hybridisation stage to releasably bind to the primer binding sites flanking each probe whereby to mitigate primer binding site interactions.

Preferably unbound probes and primers are thoroughly washed away from the bound probes following hybridisation stage and prior to analysis.

The method may be used to screen DNA, RNA and/or cDNA with appropriate probe sets. The method may be used to screen somatic and/or germline sequences. The method may be used to screen for polymorphic alterations.

The invention further provides a set of probes substantially as described above in any of the preceding seven paragraphs.

The invention may still further comprise a kit for such a method described above in any of the preceding eight paragraphs, which kit comprises a probe set generally as defined above, amplification primers and means to enable amplification and analysis of amplification product(s).

The present invention will now be described by way of example only with reference to the accompanying drawings, in which:-

Fig. 1 is a simple schematic illustration of a method according to the invention;

Fig. 2 shows a gel run produced from samples from four unrelated humans using the method according to the present invention;

Fig. 3 shows an enlargement of the central region III of the gel in Fig. 2;

Fig. 4 shows the results of quantitative analysis of the region III of the gel from one male sample (upper trace) and one female (lower trace);

Fig. 5 shows the relative signal from different probes shown plotted against probe size;

Fig. 6 shows the *EcoRV* cloning site of pZero2 and the corresponding

primer combination (PZA and PZB) used to generate probes from fragments cloned into the *EcoRV* site.

With reference to Fig. 1, there is provided a method of screening for copy number of target nucleic acid sequences in a sample of genetic material, the method comprising introducing to the sample a plurality of different genetic probes suitable to hybridise with respective target sequences and all flanked by the same or substantially the same primer binding sites, subjecting the sample to conditions favouring hybridisation of the probes to the respective sequences, and amplification of sample-bound probes using a pair of flanking primers, wherein analysis of the respective amounts of amplified probe provides for quantitative determination of the copy number of the respective nucleic acids sequences in the sample.

The method can be used to screen for genetic alterations, in particular deletions and amplifications in copy number of genetic sequences, both somatic and germline.

The sample of genetic material to be screened, which may comprise, DNA, cDNA and/or RNA, shown diagrammatically in Fig. 1 (10), is reversibly immobilised on a medium (12) such as a nylon filter.

A plurality of probes (14) is designed and prepared to seek out and hybridise with specific nucleic acid sequences, if present in the sample. The probes (14) are chosen to comprise a set of different probes each type operable to seek out a different respective nucleic acid sequence which may be sequences suspected of being involved in a particular disorder or condition for example, in the organism from which the sample has been taken. Alterations in the copy number of particular genetic sequences are known to be involved in conditions such as cancer and mental retardation, and probes specific for these sequences can be prepared and comprised in a probe set used in accordance with the present methodology for example where certain symptoms suggest these disorders or diseases may be involved.

When an appropriate probe set is created or identified, it is then introduced to the immobilised genetic material in stoichiometric excess under conditions favouring hybridisation of the probes to their respective nucleic acid sequences, if present.

Once sufficient time has elapsed to provide for hybridisation, unbound probes are washed thoroughly away and the PCR employed to amplify bound probes.

The use of the same primer pair binding sites flanking all the different probes is important to the successful operation of this method, and allows different bound probes to be amplified together with a single primer pair and thus the screening for a plurality of different loci at the same time.

Some interaction has been experienced between primer binding sites flanking different probes, but this problem has been addressed by adding to the hybridisation solution an excess of competing oligonucleotides which releasably bind to the respective primer binding sites thereby preventing such undesirable interactions whilst not adversely affecting the subsequent amplification process. The oligonucleotides are displaced once hybridisation of the probes to the sample is complete, to provide for hybridisation of the amplification primers to the binding sites.

Respective sets of probes can be made either by ligation to oligonucleotide adapters, or more simply by preparing the probes as inserts into the same site of a cloning vector, and using primers flanking the cloning site for amplification. Fig. 6 shows the cloning site of the vector pZero2 (sequence provided by InVitrogen: <http://www.invitrogen.com/vecseq-gcg/pzero2.seq>), showing the primers PZA and PZB flanking the *EcoRV* site (lowercase) used to clone blunt-ended fragments. Products amplified using these primers are therefore 59bp longer than the cloned inserts. It is thus possible to prepare sets of probes, each detecting a different locus, but all flanked by the same primer-binding sites.

It is important that a probe set is created such that each contributing probe is distinguishable, for example by virtue of its mobility through a separating gel. Following amplification, the appearance of a product of corresponding mobility acts to report the presence of a particular nucleotide sequence, and hence the presence of that sequence in the target sample. As an excess of probe is used, and amplification is restricted to the quantitative phase, the absolute amount of each product reflects its copy-number in the sample tested. Different probes may amplify with different efficiencies, but the proportional contribution from each probe will reflect its dosage in the sample and can be inferred from comparisons between different samples examined with the same probe set. As it is the retained probe that is amplified, not the sample, the gel mobility of the product is not influenced by the presence (for example) of Restriction Fragment Length Polymorphism (RFLPs) at that locus.

The following outlines experiments conducted using the method of the present invention to screen a plurality of different genetic sequences simultaneously. A set of 43 amplifiable probes was generated by cloning blunt-ended fragments into the *EcoRV* site of pZero2 (Fig. 6), followed by amplification with vector primers flanking this site, and selection of a mix of probes of different lengths. This mix included probes from 7 autosomes (1, 5, 11, 16, 17, 18 and 22), as well as the X and Y chromosomes.

The initial mix of 43 probes was subcloned from plasmids and plasmid fragments known from sequencing or hybridisation not to contain dispersed repeat elements; in the case of one of the Y-linked probes (SRY), a 1360bp fragment was amplified using primers SRYA (5'GCAGTAGAGCAGTCAGGGAG3') and SRYB (5'GGGGAGAGAAAGAAACAAGTTTG3'). Other sources of cloned genomic DNA were: chromosome 1, pJBT2 (JALA, unpublished); chromosome 5, pMS621 (Armour et al., 1996); chromosome 11, pEJ6.6 (Goldfarb et al., 1982); chromosome 16, pRN6N6A (JALA and R.M. Badge, unpublished); chromosome 17, pYNZ22 (Nakamura et al., 1988); chromosome 18, pMS440 (Armour et al., 1990); chromosome 22, pMS632c (Armour et al., 1995); chromosome X, pMS613 (Armour et al., 1990). After isolation of the genomic insert and digestion with

frequently-cutting restriction enzymes producing blunt ends (generally double digestion with *AluI* plus *HaeIII*), the resulting smaller fragments were cloned into the *EcoRV* site of pZero2 (InVitrogen) and propagated in *E.coli* TOP10 (InVitrogen). Care was taken to avoid repeat regions in DNA from minisatellite-containing clones.

With reference to Fig. 6, probes prepared by cloning blunt-ended restriction fragments from primary clones into the *EcoRV* site of pZero2 (InVitrogen) were amplified directly from bacterial cells (Sandhu et al., 1989) using flanking vector primers PZA (5'AGTAACGGCCGCCAGTGTGCTG3') and PZB (5'CGAGCGGCCGCCAGTGTGATG3'). The positions of these primers in the pZero-2 cloning site is shown in figure 6. PCR was carried out in Advanced Biotechnologies Buffer IV (75mM Tris-HCl pH8.8, 20mM (NH₄)₂SO₄, 0.01% Tween), with 0.2mM each dNTP, 1mM MgCl₂, 0.2μM each primer and 0.05U/μl Taq DNA polymerase (Advanced Biotechnologies). Reactions (generally 10μl) were subjected to 25 cycles of (95°C for 1 minute/70°C for 1 minute). Products amplified using ³³P end-labelled PZA were separated on denaturing 6% polyacrylamide/50% urea gels and probe mixes assembled such that the mobilities of all constituent probes were distinct.

Sample DNA for immobilisation was prepared in an initial volume of less than 5μl, denatured by addition of 1μl 1M NaOH, and spotted onto a nylon filter (MSI MAGNA, approximate dimensions 2mm x 4mm), 1μl at a time, allowing the solution to dry between applications. When all the DNA had been added the DNA was irreversibly bound to the filter using U.V. irradiation. Since numerous filters were to be used in the same hybridisation, individual marks cut into the edges and corners made clear the identity of each filter.

Filters were prehybridised in 1ml of prehybridisation solution (0.5M sodium phosphate pH7.2, 7% SDS, 0.1mg/ml alkali-denatured herring sperm DNA) at 65°C overnight, and before hybridisation this was exchanged for 200μl of prehybridisation solution with the addition of denatured human Cot-1 DNA (Gibco BRL) to a final concentration of 10μg/ml. Probe mixtures containing

about 100pg of each sequence in 1µl were mixed with 7µg *E.coli* DNA (DH5α DNA, digested with *Hae*III) and 1µg human Cot-1 DNA (Gibco-BRL), and denatured by adding 2µl 1M NaOH. After 1 minute at 37°C, the probe mixture was placed on ice, neutralized by adding 3µl 1M NaH₂PO₄, and added to the hybridisation solution.

Hybridisation was left to proceed at 65°C overnight, and the filters were thoroughly washed at 65°C in (a) two 1ml changes of prehybridisation solution, (b) 200ml 1x SSC/1% SDS and (c) 500ml 0.1x SSC 0.1% SDS. Washed filters were then transferred to individual 50µl amplification reactions (constituents as for probe amplification), and bound DNA amplified for 5 cycles of 95°C 1 minute/70°C 1 minute.

This low-level preamplified solution was then used to seed further 10µl amplifications using ³³P 5' end-labelled primer PZA. Labelled PCR products were given a final incubation at 72°C for 20 minutes (to drive to completion the addition of untemplated A at the 3' ends), an equal volume of formamide dye mix (98% formamide, 10mM EDTA pH 8.0, 1mg/ml xylene cyanol FF, 1mg/ml bromophenol blue) added, and the DNA denatured at 100°C for 2 minutes and then placed on ice. From this denatured sample, 4µl was loaded into a well of a 6% polyacrylamide/50% urea gel in 0.5x TBE, and the gel run at 90W (to maintain a gel temperature of about 45°C) until the xylene cyanol dye was close to the end of the gel. Gels were fixed in 10% methanol/10% acetic acid, dried, and radioactivity detected either by standard autoradiography or (for quantitative analysis) using ImageQuant software analysis on data captured by a storage phosphorimager screen (Molecular Dynamics).

The screening results of hybridising this probe set with DNA from one male and three females is shown in figure 2. The autosomal loci give comparable signals, between all individuals; while there is probe-to-probe variation in absolute intensity, the relative contribution of each band to the whole is highly reproducible. By contrast, the Y-derived probes only give strong signals in the hybridisation with male DNA. The relative signal from the X-

linked probes is reduced by about 50% in the male (figures 3 and 4).

In figure 5, the normalised relative signal from each probe is shown plotted against the length of the probe; signal intensities are normalised relative to the mean value for that probe among all individuals tested for autosomal loci, among all the females for X-linked probes and relative to the male for Y-linked probes. The boxed points are the points for X-linked loci in the male (about 50% signal intensity) and for Y-linked probes in females (close to zero). This experiment models the expected detection of homozygous deletions (Y-linked loci in females) or hemizygous deletions (X-linked loci in males). In the size range 140-600bp the signal appears to reflect copy number variation consistently.

Nearly all the amplified bands seen after hybridisation corresponded in size with the expected probes. There were however three bands (marked with asterisks on figure 2) which did not correspond to probes of known origin; the largest of these is clearly Y-linked. The simplest explanation for the appearance of these bands is that they are the result of contamination of the probe mix by other probes made at the same time. Since (if hybridisation has gone to near completion) the intensity of the band recovered from a target DNA will depend on its copy-number in the target DNA, and not on its concentration in the probe mix, even relatively minor contamination with additional probes could result in the appearance of unexpected bands in the amplified product at intensities equal to the predicted bands. Probe fragments corresponding to two of these additional bands are detectable as components of the initial probe mix. The importance of avoiding even minor contamination with additional probes represents an advantage of isolating and storing the probes as clones (rather than PCR products), since uncontaminated probe preparations can be reconstituted from colony-purified clones.

The efficiency of this method derives from its ability to examine numerous loci simultaneously (in this experiment, 43 probes). In the format used here, in which radiolabelled probes are resolved using denaturing

polyacrylamide gel electrophoresis, the number of probes which can be analysed simultaneously is limited only by the resolving power of the gel. Since probes in the size range 140-600bp have been used successfully in this experiment, careful size-selection of probes could lead to probe sets containing more than a hundred probes, which might (for example) be used as chromosome-specific subsets to analyse copy number at megabase resolution.

Moreover, greater resolution may be achieved using fluorescently-labelled probes and apparatus and software designed for automated sequence analysis. This would not only have the advantage of single-base resolution over a larger range (and hence the possibility of more probes per lane), but could also use multiple fluors in a single track. Thus if (for example) four sets of each of 100 probes were assembled, the probes of each set flanked by different primer binding sites (same within a set), they could be hybridised with the same target, and after the initial recovery of bound probes and low-level preamplification using all primer pairs, each set could be selectively amplified with its own primer pair (one fluorescent), and the four resulting sets of fluorescent products mixed and run in the same lane. Similarly, if the same primer was available with different fluorescent labels, then a direct comparison between two samples (for example tumour versus normal from the same person) could be made by labelling the products from each with different fluors and running in the same lane of the gel.

In the present format, the high resolution of polyacrylamide gel electrophoresis allows the simultaneous differentiation of products from many different loci, but even using all the available "slots" with judicious assembly of probe sets, this format probably limits the number of probes flanked by a given primer pair which can be resolved in one lane to about 500, and hence if four probe sets with different flanking primers can be used simultaneously, fewer than 2000 probes can be examined simultaneously in this test. It might be possible to augment the resolution of this analysis by using formats for the quantitative detection of amplified probe which do not rely on gel electrophoresis. One alternative would be to use recovered probes to label

arrays of the clones from which the probes themselves were produced; a procedure analogous to array-CGH (Pinkel et al., 1998), but with the added efficiency that the method according to the present invention the MAPH has already produced proportionate amounts of the relevant single-copy probes from the genomic targets, reducing the complexity of the mixture (for increased speed of hybridisation) and by avoiding multi-copy sequences, presumably allowing quantification with a greater signal:noise ratio.

The experiments explained here have used genomic DNA as the target nucleic acid, but an extension of this method would be to use probes to probe RNA (or cDNA) for highly parallel analysis of gene expression. Current RT-PCR based methods for quantification of gene expression do not allow the simple analysis of numerous loci simultaneously.

The method could be used to screen for alterations to a plurality of sequences relating to different genes, and/or sequences within a target gene e.g. screen for alterations in exon copy number within a eukaryotic gene.

Using the method according to the present invention to detect deletions and amplifications in (for example) idiopathic mental retardation (IMR) assumes that the absence of a locus is abnormal, rather than resulting from a polymorphism. This can be most simply investigated by analysis of the parents in cases of IMR, but it remains possible that wider application of this technique will uncover loci at which there are presence/absence polymorphisms. If enough (sufficiently informative) loci of this kind were available, then assembly of such probes into a mutually compatible "polymorphic set" would allow simultaneous genotyping at (say) 50-100 loci in a single lane of a sequencing gel; combined with analysis of mixed DNA samples, such a system could provide very high throughput for genotyping without the need for investment in expensive or complicated equipment.

Whilst endeavouring in the foregoing specification to draw attention to those features of the invention believed to be of particular importance it should

be understood that the Applicant claims protection in respect of any patentable feature or combination of features hereinbefore referred to and/or shown in the drawings whether or not particular emphasis has been placed thereon.

CLAIMS

1. A method of screening for copy number of target nucleic acid sequences in a sample of genetic material (10), the method comprising introducing to the sample (10) a plurality of different genetic probes (14) suitable to hybridise with respective target sequences and all flanked by the same or substantially the same primer binding sites, subjecting the sample (10) to conditions favouring hybridisation of the probes (14) to their respective sequences, and amplification of sample-bound probes (14) using a pair of primers, wherein analysis of the respective amounts of amplified probe (14) provides for quantitative determination of the copy number of the respective nucleic acid sequences in the sample (10).
2. A method according to claim 1, characterised in that each probe (14) is distinguishable from the other(s), for example by having distinguishing mobility characteristics through a separating gel.
3. A method according to claim 1 or 2, characterised in that the plurality of different probes (14) comprises a predetermined set of different probes (14) each chosen to be specific for a respective target nucleic acid sequence.
4. A method according to claim 3, characterised in that the set comprises probes (14) suitable to screen a plurality of different nucleic acid sequences simultaneously or substantially simultaneously such that determination of the quantity of each probe product produced enables quantitative determination of the copy number of the respective sequences in the sample.
5. A method according to claim 4, characterised in that the polymerase chain reaction is used to determine the quantity of each probe product produced.
6. A method according to any of the preceding claims, characterised in that the method is used to screen sequences of different genes or different

sequences within a gene, such as different exons in a eukaryotic gene.

7. A method according to any of the preceding claims, characterised in that the method is used to detect genetic alterations such as genetic deletions (reduction in sequence copy number) and genetic amplification (increase in sequence copy number).
8. A method according to any of the preceding claims, characterised in that the genetic material (10) is immobilised prior to hybridisation, such that hybridised flanking primers are likewise immobilised.
9. A method according to any of the preceding claims, characterised in that an excess of probes (14) is used.
10. A method according to any of the preceding claims, characterised in that probes (14) labelled for ready identification are used.
11. A method according to claim 10, characterised in that probes labelled with fluorescent labels are used.
12. A method according to any of claims 3 to 11, characterised in that more than one set of probes (14) is used, either simultaneously or sequentially.
13. A method according to claim 12, characterised in that the flanking primer pairs are the same for each set of probes (14).
14. A method according to claim 12, characterised in that the flanking primer pairs are different for each set of probes (14).
15. A method according to any of the preceding claims, characterised in that the method comprises means to obviate or mitigate hybridisation between primer binding sequences.

16. A method according to claim 15, characterised in that competing oligonucleotides are introduced to the sample preferably during the hybridisation stage to releasably bind to the primer binding sites flanking each probe whereby to mitigate primer binding site interactions.

17. A method according to any of the preceding claims, characterised in that unbound probes (14) and primers are thoroughly washed away from the bound probes (14) following hybridisation stage and prior to analysis.

18. A method according to any of claims 3 to 17, characterised in that the method is used to screen DNA, RNA and/or cDNA with appropriate probe sets.

19. A method according to claim 18, characterised in that the method is used to screen somatic and/or germline sequences.

20. A method according to claim 18 or 19, characterised in that the method is used to screen for polymorphic alterations.

21. A set of probes (14) substantially as described above in any of the preceding claims.

22. A kit for such a method described above in any of the preceding claims, which kit comprises a probe set (14) generally as defined above, amplification primers and means to enable amplification and analysis of amplification product(s).

23. A method of screening for copy number of target nucleic acid sequences in a sample of genetic material substantially as hereinbefore described with reference to the accompanying examples and drawings.

24. A set of probes substantially as hereinbefore described with reference to the accompanying examples and drawings.

25. A kit for a method of screening for copy number of target nucleic acid sequences in a sample of genetic material substantially as hereinbefore described with reference to the accompanying examples and drawings.

26. Any novel subject matter or combination including novel subject matter disclosed herein, whether or not within the scope of or relating to the same invention as any of the preceding claims.

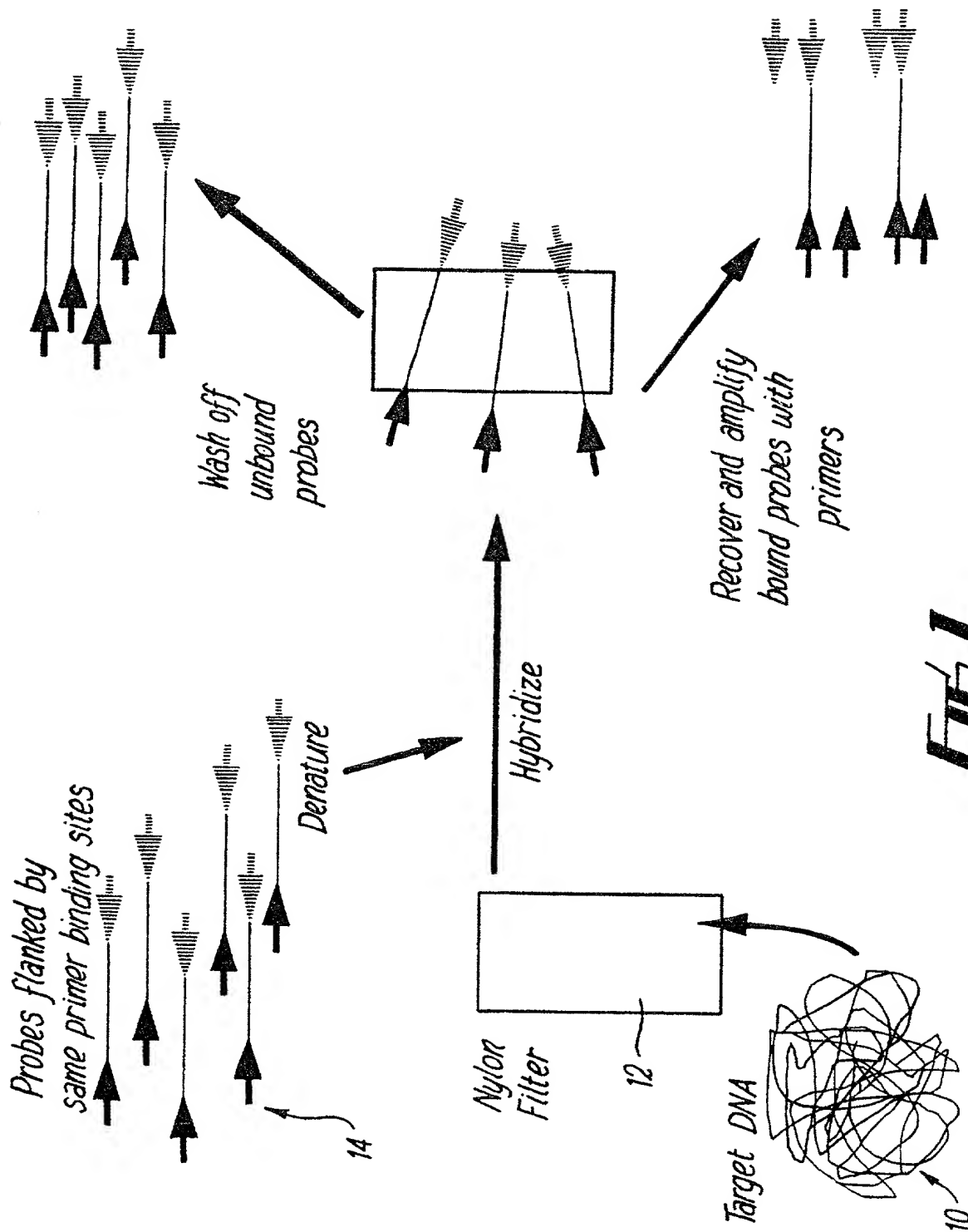
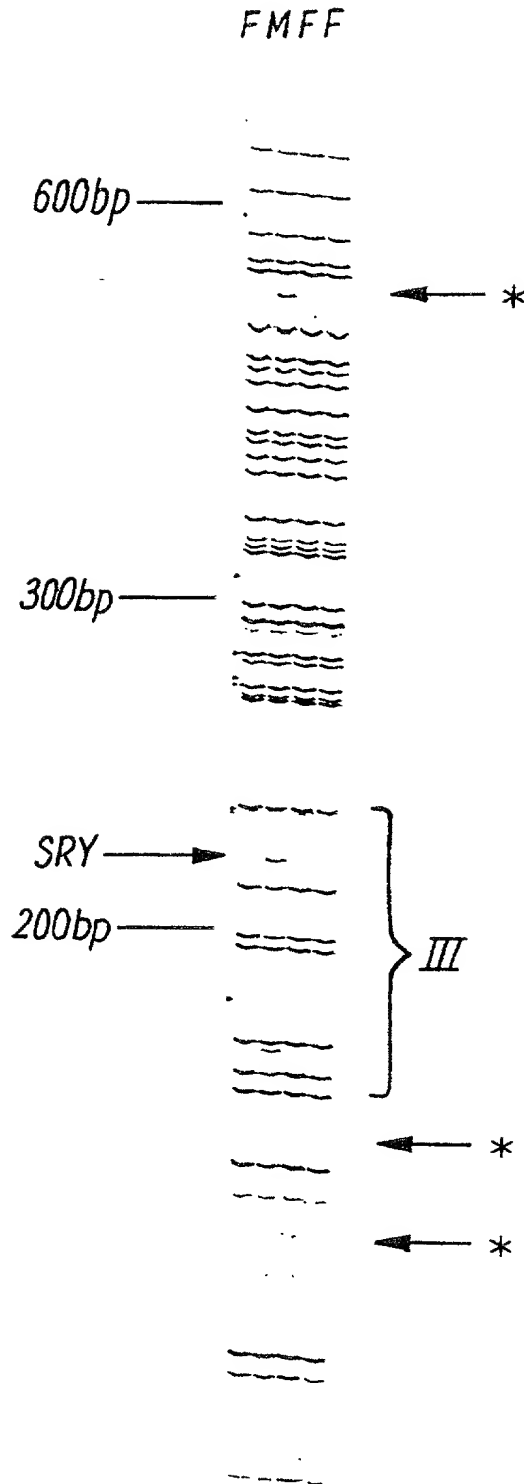
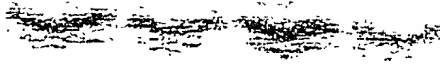



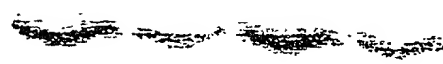
FIG. 1

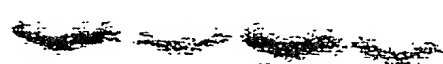
**Fig. 2**

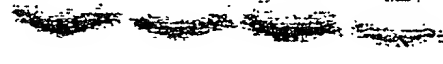
F M F F

22 → 

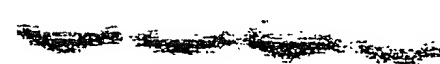
Y → 

X → 

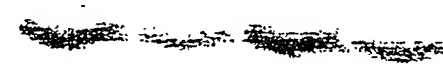
X → 

17 → 

15

22 → 

Y → 

X → 

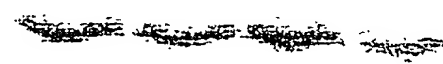
17 → 

FIG. 3

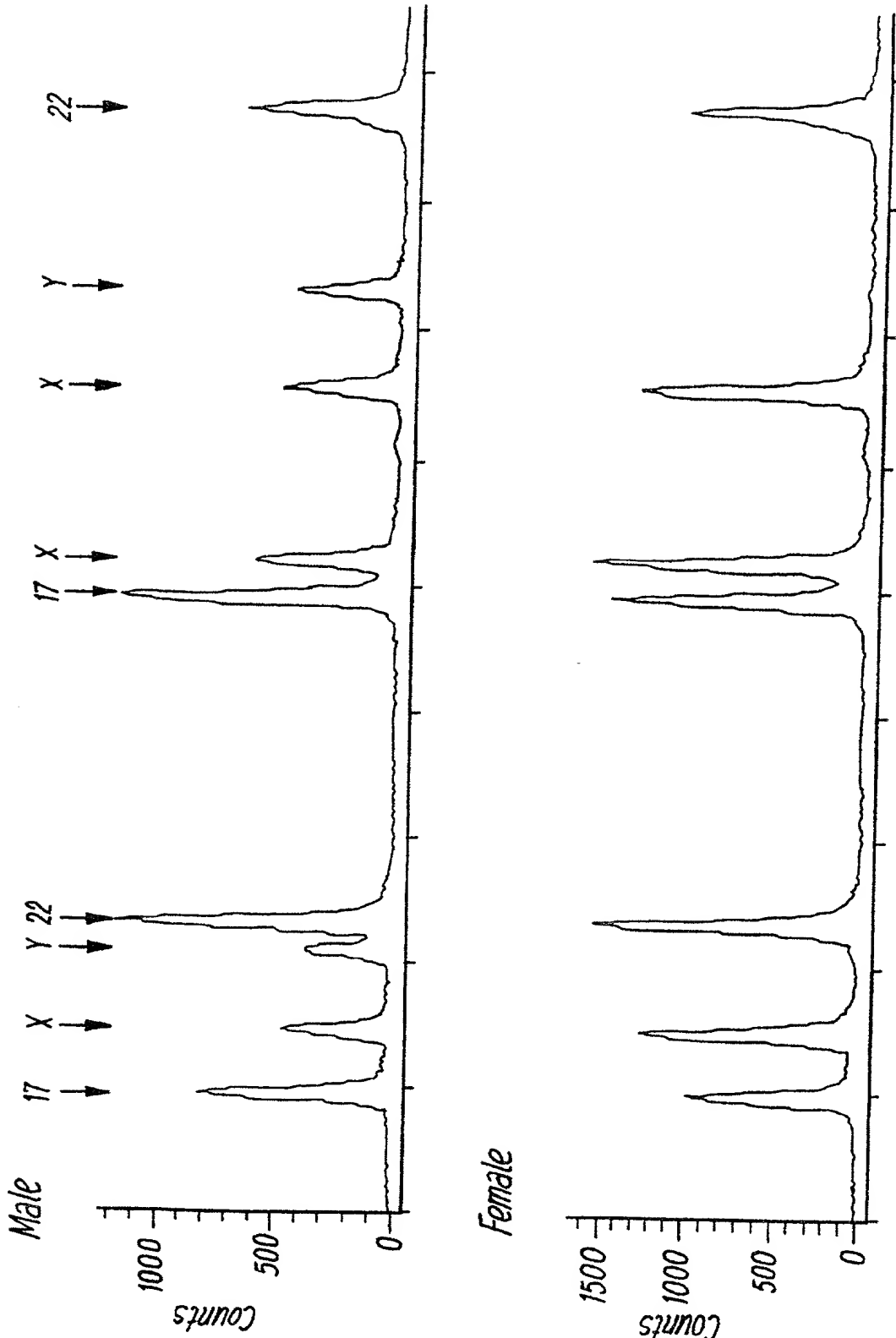
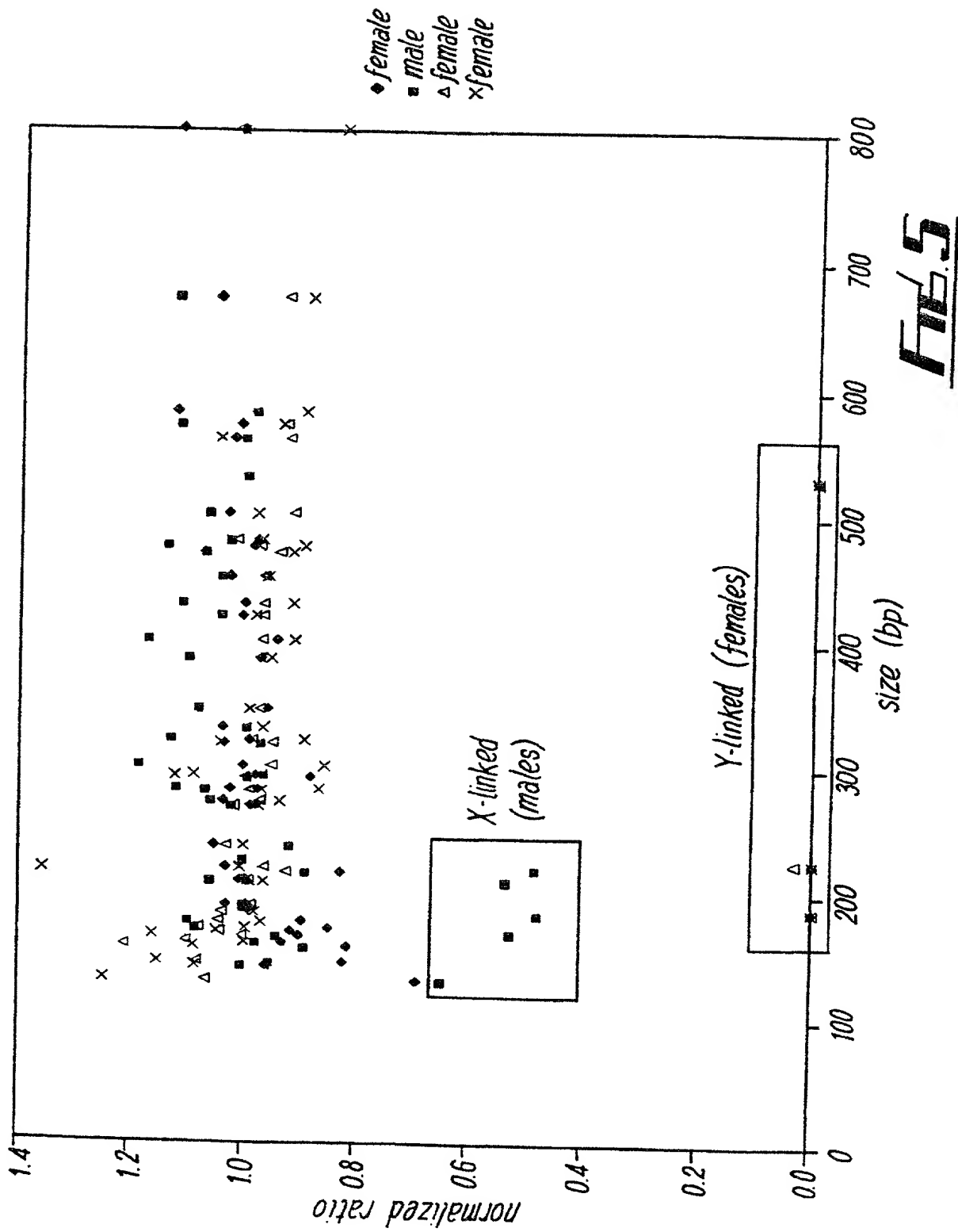


Fig. 4

1877-1878 1879-1880 1881-1882 1883-1884 1885-1886 1887-1888 1889-1890 1891-1892 1893-1894 1895-1896 1897-1898 1899-1900 1901-1902 1903-1904 1905-1906 1907-1908 1909-1910 1911-1912 1913-1914 1915-1916 1917-1918 1919-1920 1921-1922 1923-1924 1925-1926 1927-1928 1929-1930 1931-1932 1933-1934 1935-1936 1937-1938 1939-1940 1941-1942 1943-1944 1945-1946 1947-1948 1949-1950 1951-1952 1953-1954 1955-1956 1957-1958 1959-1960 1961-1962 1963-1964 1965-1966 1967-1968 1969-1970 1971-1972 1973-1974 1975-1976 1977-1978 1979-1980 1981-1982 1983-1984 1985-1986 1987-1988 1989-1990 1991-1992 1993-1994 1995-1996 1997-1998 1999-2000 2001-2002 2003-2004 2005-2006 2007-2008 2009-2010 2011-2012 2013-2014 2015-2016 2017-2018 2019-2020 2021-2022 2023-2024 2025-2026 2027-2028 2029-2030 2031-2032 2033-2034 2035-2036 2037-2038 2039-2040 2041-2042 2043-2044 2045-2046 2047-2048 2049-2050 2051-2052 2053-2054 2055-2056 2057-2058 2059-2060 2061-2062 2063-2064 2065-2066 2067-2068 2069-2070 2071-2072 2073-2074 2075-2076 2077-2078 2079-2080 2081-2082 2083-2084 2085-2086 2087-2088 2089-2090 2091-2092 2093-2094 2095-2096 2097-2098 2099-2100 2101-2102 2103-2104 2105-2106 2107-2108 2109-2110 2111-2112 2113-2114 2115-2116 2117-2118 2119-2120 2121-2122 2123-2124 2125-2126 2127-2128 2129-2130 2131-2132 2133-2134 2135-2136 2137-2138 2139-2140 2141-2142 2143-2144 2145-2146 2147-2148 2149-2150 2151-2152 2153-2154 2155-2156 2157-2158 2159-2160 2161-2162 2163-2164 2165-2166 2167-2168 2169-2170 2171-2172 2173-2174 2175-2176 2177-2178 2179-2180 2181-2182 2183-2184 2185-2186 2187-2188 2189-2190 2191-2192 2193-2194 2195-2196 2197-2198 2199-2200 2201-2202 2203-2204 2205-2206 2207-2208 2209-2210 2211-2212 2213-2214 2215-2216 2217-2218 2219-2220 2221-2222 2223-2224 2225-2226 2227-2228 2229-2230 2231-2232 2233-2234 2235-2236 2237-2238 2239-2240 2241-2242 2243-2244 2245-2246 2247-2248 2249-2250 2251-2252 2253-2254 2255-2256 2257-2258 2259-2260 2261-2262 2263-2264 2265-2266 2267-2268 2269-2270 2271-2272 2273-2274 2275-2276 2277-2278 2279-2280 2281-2282 2283-2284 2285-2286 2287-2288 2289-2290 2291-2292 2293-2294 2295-2296 2297-2298 2299-2300 2301-2302 2303-2304 2305-2306 2307-2308 2309-2310 2311-2312 2313-2314 2315-2316 2317-2318 2319-2320 2321-2322 2323-2324 2325-2326 2327-2328 2329-2330 2331-2332 2333-2334 2335-2336 2337-2338 2339-2340 2341-2342 2343-2344 2345-2346 2347-2348 2349-2350 2351-2352 2353-2354 2355-2356 2357-2358 2359-2360 2361-2362 2363-2364 2365-2366 2367-2368 2369-2370 2371-2372 2373-2374 2375-2376 2377-2378 2379-2380 2381-2382 2383-2384 2385-2386 2387-2388 2389-2390 2391-2392 2393-2394 2395-2396 2397-2398 2399-2400 2401-2402 2403-2404 2405-2406 2407-2408 2409-2410 2411-2412 2413-2414 2415-2416 2417-2418 2419-2420 2421-2422 2423-2424 2425-2426 2427-2428 2429-2430 2431-2432 2433-2434 2435-2436 2437-2438 2439-2440 2441-2442 2443-2444 2445-2446 2447-2448 2449-2450 2451-2452 2453-2454 2455-2456 2457-2458 2459-2460 2461-2462 2463-2464 2465-2466 2467-2468 2469-2470 2471-2472 2473-2474 2475-2476 2477-2478 2479-2480 2481-2482 2483-2484 2485-2486 2487-2488 2489-2490 2491-2492 2493-2494 2495-2496 2497-2498 2499-2500 2501-2502 2503-2504 2505-2506 2507-2508 2509-2510 2511-2512 2513-2514 2515-2516 2517-2518 2519-2520 2521-2522 2523-2524 2525-2526 2527-2528 2529-2530 2531-2532 2533-2534 2535-2536 2537-2538 2539-2540 2541-2542 2543-2544 2545-2546 2547-2548 2549-2550 2551-2552 2553-2554 2555-2556 2557-2558 2559-2560 2561-2562 2563-2564 2565-2566 2567-2568 2569-2570 2571-2572 2573-2574 2575-2576 2577-2578 2579-2580 2581-2582 2583-2584 2585-2586 2587-2588 2589-2590 2591-2592 2593-2594 2595-2596 2597-2598 2599-2600 2601-2602 2603-2604 2605-2606 2607-2608 2609-2610 2611-2612 2613-2614 2615-2616 2617-2618 2619-2620 2621-2622 2623-2624 2625-2626 2627-2628 2629-2630 2631-2632 2633-2634 2635-2636 2637-2638 2639-2640 2641-2642 2643-2644 2645-2646 2647-2648 2649-2650 2651-2652 2653-2654 2655-2656 2657-2658 2659-2660 2661-2662 2663-2664 2665-2666 2667-2668 2669-2670 2671-2672 2673-2674 2675-2676 2677-2678 2679-2680 2681-2682 2683-2684 2685-2686 2687-2688 2689-2690 2691-2692 2693-2694 2



```

primer PZA
AGTAACGGCCGCCAGTGTGCTG          EcoRV
GGATCCACTAGTAACGGCCGCCAGTGTGCTGGAATTCTGCAGatatacCATCACACTGGCGCGCTCGAGCA
+-----+-----+-----+-----+-----+-----+-----+-----+-----+
CCTAGGTGATCATTGCCGGGTCACACGACCTTAAGACGTctatagGTAGTGTGACCGCGCGGAGCTCGT
                                GTAGTGTGACCGCGCGGAGC
                                primer PZB

```

FILE

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (Includes Reference to PCT International Applications)

 ATTORNEY'S DOCKET NUMBER
16-007

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

GENETIC SCREENING

the specification of which (check only one item below):

☐ is attached hereto.

☒ was filed as United States application

 Serial No. 09/914,811

 on August 30, 2001

and was amended

on _____ (if applicable).

☒ was filed as PCT international application

 Number PCT/GB00/00761

 on 3 March 2000 (03.03.00)

and was amended under PCT Article 19

on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119;

COUNTRY (if PCT indicate "PCT")	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 USC 119
PCT	PCT/GB00/00761	3 March 2000 (03.03.00)	5 March 1999 (05.03.99)
GREAT BRITAIN	9904991.3	05 March 1999 (05.03.99)	



09/914,811 03.03.00

US

Combined Declaration for Patent Application and Power of Attorney (Continued)
 (Includes Reference to PCT International Applications)

 ATTORNEY'S DOCKET NUMBER
 16-007

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120:

U.S. APPLICATIONS		STATUS (Check One)		
U.S. APPLICATION NUMBER	U.S. FILING DATE	PATENTED	PENDING	ABANDONED
PCT APPLICATIONS DESIGNATING THE U.S.				
PCT APPLICATION NO.	PCT FILING DATE	U.S. SERIAL NUMBERS ASSIGNED (if any)		
PCT/GB00/00761	03.03.00			

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (List name and registration number):
 T.E. Fisher, Reg. No. 18,271; L.L. Heinke, Reg. No. 19,471; J.G. Waterson, Reg. No. 20,180; L.J. Raney, Reg. No. 23,078;
 J.R. Hlavka, Reg. No. 28,076; S.J. Schultz, Reg. No. 29,108; R.A. Sharpe, Reg. No. 34,722; G.L. Pinchak, Reg. No. 37,627;
 P.A. Serbinowski, Reg. No. 34,429; K.J. Smith, Reg. No. 45,115; William Johnston, Reg. No. 47,687; Robert J. Diaz, Reg. No. 44,655 and Jennifer Nock Hinton, Reg. No. 47,653.

Send Correspondence to: Richard A. Sharpe
 Watts, Hoffmann, Fisher & Heinke Co.
 P.O. Box 99839
 Cleveland, Ohio 44199-0839

Direct Telephone Calls to:
 (name and telephone number)

216/241-6700

FULL NAME OF INVENTOR	FAMILY NAME <u>ARMOUR</u>	FIRST GIVEN NAME <u>John</u>	SECOND GIVEN NAME <u>Anthony</u>
RESIDENCE & CITIZENSHIP	CITY <u>Nottingham</u>	STATE OR FOREIGN COUNTRY <u>United Kingdom</u> <u>GBX</u>	COUNTRY OF CITIZENSHIP <u>Great Britain</u>
POST OFFICE ADDRESS	POST OFFICE ADDRESS <u>Flat 22, Barker Gate</u>	CITY <u>Nottingham</u>	STATE & ZIP CODE/COUNTRY <u>NG1 1JU United Kingdom</u>
FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE & ZIP CODE/COUNTRY

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201

SIGNATURE OF INVENTOR 202

SIGNATURE OF INVENTOR 203

DATE

DATE

DATE

 OIPE JCTP
 JAN 03 2002
 PATENT & TRADEMARK

 RECEIVED
 JAN 03 2002